



**ESTIMATION OF EXTRACELLULAR LIPOLYTIC ENZYME ACTIVITY BY  
THERMOPHILIC *BACILLUS* SP. ISOLATED FROM ARID AND SEMI-ARID  
REGION OF RAJASTHAN, INDIA**

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**ABSTRACT**

Thermophilic organisms can be defined as, micro-organisms which are adapted to survive at high temperatures. The enzymes secreted by thermophilic bacteria are capable of catalyzing biochemical reactions at high temperatures. Thermophilic bacteria are able to produce thermostable lipolytic enzymes (capable of degradation of lipid) at temperatures higher than mesophilic bacteria. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are quite beneficial in terms of discovering thermostable lipase enzymes. Due to great temperature fluctuation in hot arid and semi-arid region of Rajasthan, this area could serve as a good source for new thermophilic lipase producing bacteria with novel industrially important properties. The main objective of this research is the isolation and estimation of industrially important thermophilic lipase enzyme produced by thermophilic bacteria, isolated from arid and semi-arid region of Rajasthan. For this research purpose soil samples were collected from Churu, Sikar and Jhunjunu regions of Rajasthan. Total 16 bacterial strains were isolated and among only 2 thermostable lipolytic enzyme producing bacteria were characterized. The thermostable lipolytic enzyme was estimated by qualitative and quantitative experiments. The isolates were identified as *Bacillus* sp. by microscopic, biochemical and molecular characterization. The optimum enzyme activity was

observed at pH 8, temperature 60°C and 6% salt concentrations at 24 hrs time duration. Lipolytic enzyme find useful in a variety of biotechnological fields such as food and dairy (cheese ripening, flavour development), detergent, pharmaceutical (naproxen, ibuprofen), agrochemical (insecticide, pesticide) and oleochemical (fat and oil hydrolysis, biosurfactant synthesis) industries. Lipolytic enzyme can be further used in many newer areas where they can serve as potential biocatalysts.

**Keywords:** Thermophiles, Thermostable enzyme, Lipolytic enzyme, Arid region, Semi-arid region, *Bacillus* sp.

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## INTRODUCTION

The search for extremophilic micro-organisms is one of the main reason for obtaining thermostable enzymes useful for industrial applications. There are several advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes (**Kristinsson, 1989**). The main advantage is that as the temperature of the process is increases, the rate of reaction also increases; as a 10°C increase in temperature, approximately doubles the reaction rate, which in turn least amount of enzymes requiries for the production formation (**Haki and Rakshit, 2003**). The thermostable enzymes are able to tolerate higher temperatures, which give longer half-life stability to the enzyme. Thermostable enzymes are of great demand and concern due to their high stability, which makes them very helpful for several industrial processes and applications. It is not only their thermostability but also their greater stability under other extreme conditions or stress like high or low pH, low water availability etc. These are the most important and great significant enzymes in present day biotechnology. Although they can be derived from several sources, such as plants, animal and micro-organisms; enzymes from microbial sources generally meet industrial demands. The use of higher temperature in industrial processes reduces the risk of contamination caused by other mesophilic micro-organisms and simultaneously thermostable enzymes (thermozymes) are very useful useful in the processing of lower viscosity fluids (at higher temperature viscosity is usually reduced) and also reduces filtration, centrifugation and costs of pumping. The transfer rates of heat and mass are also improved at high temperature, diffusion rates are higher and mass transfer is less limiting. As temperature increases more substrate dissolved which can shift the equilibrium to a higher product yield in comparison to other enzymes of low temperature stability.

Lipases are ubiquitous enzymes (**Brockerhoff and Jensen, 1974; Borgstrom and Brockman, 1984; Desnuelle and Sjostrom, 1986; Wooley and Petersen, 1994**) which are found in animals, plants (**Huang, 1993; Mukherjee and Hills, 1994**) fungi (**Iwai and Tsujisaka, 1984**) and bacteria (**Brune and Goetz, 1992; Jaeger et al., 1994; Jaeger and Reetz, 1998**). Lipolytic enzymes occur widely in nature but only microbial lipolytic enzymes are commercially significant (**Sharma et al., 2001**). Lipolytic enzymes are a versatile group of enzymes and also express other activities like phospholipase, isophospholipase, cholesterol esterase, cutinase, amidase and other esterase type of activities (**Svendsen, 2000**). Lipolytic enzymes are hydrolytic enzymes which hydrolyse triglycerides to free fatty acids and glycerol (**Sangeetha et al., 2011**). Recently several thermophilic lipases have been purified and characterized from thermophilic *Bacillus* sp. (**Sidhu, et al., 1998; Sharma et al., 2002**) like *Bacillus thermoleovorans* (**Markossian et al., 2000; Lee et al., 2001**), *Bacillus stearothermophilus* (**Sinchaikul et al., 2001**) and *Bacillus circulans* (**Kademi et al., 2000**). Thermostable lipolytic enzymes from the archeon *Sulfolobus acidocaldarius*, *Clostridium saccharolyticum*, *Pyrococcus furiosus* and many *Pseudomonas* sp. have also been reported (**Niehaus et al., 1999**).

Lipases (triacylglycerol acylhydrolases) belong to the class of serine hydrolases and does not require any co-factor. The natural substrate of Lipolytic enzymes are triacylglycerols, which is having very low water solubility. These enzymes catalyze the hydrolysis of ester bonds under natural conditions and convert triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. Lipolytic enzymes have attracted much attention during the last decade due to the diversity in their applications. The enantioselective and regioselective nature of this enzyme have been utilized for the resolution of chiral drugs, fat modification, synthesis of cocoa butter constituents, bio-fuels, synthesis of personal care products and flavor enhancers (**Gerhartz, 1990**). More than 70 lipases, including at least 47 different lipases from bacteria have been cloned and sequenced (**Jaeger et al., 1999**). The active site of  $\alpha/\beta$  hydrolases contains a catalytic triad which consists of conserved serine, aspartic or glutamic acid and histidine residues. These enzymes share a common catalytic mechanism and structure and also show some similarity with each other at the amino acid level (**Jaeger et al., 1994**). Lipolytic enzymes are used in fat hydrolysis or as catalysts in synthetic organic chemistry (**Jaeger et al., 1999**), for an example around 1000 tons of lipase are used in detergents for fat degradation every year.

Lipolytic enzymes from organisms such as *Serratia marcescens* are used on a large scale in the pharmaceutical industry for the production of drugs such as Diltiazem (**Jaeger et**

*al.*, 1999). Therefore, prospecting for novel lipase genes having interest for both academic and industrial reasons. The novel lipase genes are difficult to isolate due to some reason including toxicity of expression to heterologous, requirement of helper proteins for functional lipase expression and low homology observed between different lipase genes which makes them difficult targets for PCR cloning (**Philip et al., 2002**).

Lipolytic enzymes are a versatile group of enzymes and often express other activities like isophospholipase, phospholipase, amidase, cholesterol esterase and cutinase etc. (**Svendsen, 2000**). They have a number of unique characteristics like stereospecificity, regiospecificity substrate, specificity and ability to catalyse a heterogeneous reaction at the interface of water soluble and water insoluble systems (**Sharma et al., 2002**). Lipolytic enzymes of fungal origin has also been commercialized (**Yavuz, 2003**).

We selected Arid and Semi Arid region of Rajasthan for study of great temperature fluctuation; these areas could serve as a good source for new thermophilic micro-organisms with novel industrially important properties. The aim of present study is the isolation and identification of industrially important extracellular lipolytic enzyme producing thermophilic bacteria from Arid and Semi arid region of Rajasthan and further optimization of physical and cultural parameters which affect the lipolytic enzyme production and activity. The isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new industrial enzymes.

## **MATERIAL AND METHODS**

Soil samples were collected in sterile containers from Churu, Jhunjunu and Sikar (Shekhawati Region), located in the state of Rajasthan, India. Sampling was done at the temperature between 50°C to 55°C and pH of the soil was around 7.5. The collected soil samples were serially diluted up to  $10^{-1}$  to  $10^{-6}$  and spreaded on agar plates followed by incubation at 50°C.

### **Screening of lipolytic enzyme producing bacteria**

Several methods have been proposed for screening of lipase production. These methods either directly use the microorganism under study (**Nair and Kumar, 2007**) or measure lipolytic activity in the crude or purified culture preparations (**Singh et al., 2010**). In present investigation author followed the screening of lipase producing bacteria on tributyrin

agar plates (Lawrence *et al.*, 1967). The composition of tributyrin agar (g/l) was, peptone from meat-2.5, peptone from casien-2.5, Yeast extract-3.0, agarose-12.0 and tributyrin 10 ml, pH-7. Each culture was streaked onto the tributyrin agar plate and incubated at 50°C for 2 days. Plate detection method was used for the observation of lipolysis through the presence of clear lipolysis zones around bacterial streaks on tributyrin agar plates.

### **Identification of lipolytic enzyme producing bacteria**

Isolates were observed under the microscope and the bacterial colonies were noted on the basis of their color, size, shape, margins and pigmentation.

### **Microscopic observation**

An attempt was made to identify the bacterial strains on the basis of Gram staining, endospore staining, capsule staining and motility test.

### **Biochemical characterization**

Biochemical characterization of bacterial isolates was done by various biochemical tests like Indole test, MR-VP test, Simmons citrate, Starch hydrolysis, H<sub>2</sub>S production, Catalase, Oxidase, Urease, Nitrate reduction test and Gelatin hydrolysis test.

### **Molecular characterization DNA preparation and PCR amplification**

Genomic DNA was extracted from the bacterial isolates by CTAB (Cetyltrimethylammonium Bromide) method (Ausubel *et al.*, 1994). In this method bacterial pellet was dissolved in CTAB extraction buffer (Tris-HCl-0.2M, Disodium EDTA-0.02M, NaCl-1.4M, CTAB-2% and  $\beta$ -merceptoetanol-2%). CTAB is a nonionic detergent which complexes with carbohydrates and other macromolecules such as proteins and other cell wall debris and causes their precipitation on phenol extraction. Nucleic acid upon centrifugation come in the aqueous phase and precipitated by ice cooled isopropanol and ethanol (76%). For removal of RNA, isolated DNA was mixed with DNase free RNase (1 $\mu$ l/ml) and incubated at 37°C for 1 hour. DNA was air dried at room temperature for overnight, subsequently dissolved in appropriate volume of TE buffer and stored at -20°C till further use.

A PCR amplification was done by universal 16S rDNA primers (Forward Primer: 5'- TGCGGCTGGATCCCCTCCTT-3', Reverse Primer: 5'-CCGGGTTTCCCCATTCGG-3') and thermocycler was programmed as (denaturation at 94°C for 2 min -30 cycles, annealing at 55°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 5 min). PCR products were identified by agarose gel electrophoresis in 1% agarose gel (Marchesi *et al.*, 1998).

### **16S rDNA sequencing and data analysis**

ABI automated sequencer was used for the sequence analysis. Sequencing of DNA was performed by 1500 bp PCR product and then two 16S rDNA sequences were aligned and compared with other 16S rDNA genes in the GenBank by using the NCBI Basic Local Alignment Search Tools (BLAST).

### **Assay of lipase Activity**

The isolated lipase producing bacteria were inoculated in 50 ml production medium (starch-15, peptone-15, NH<sub>4</sub>Cl-2.5, MgSO<sub>4</sub>-0.7, K<sub>2</sub>HPO<sub>4</sub>-2.0 (g/l) and Olive oil 2%) in triplicate (one control and three replicates /sample) and incubated at 50°C for 36 hours on a rotary shaker at 200 rpm. The cultures were centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was used as the source of crude lipase enzyme because cell debris settled down and crude enzyme separated as supernatant. Supernatant was filtered through a 0.22µm pore size membrane filter. This filtrate was examined for lipase activity using lipase kit (Spinreact Spain, SP1001275). Lipase in presence of colipase, desoxycholate and calcium ions, hydrolyses the substrate 1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester. The rate of methylresorufin formation, measured photometrically at 580 nm, is proportional to the catalytic concentration of lipase present in the sample. One unit of lipase activity was defined as the amount of enzyme required for the consumption of 1 µmol substrate per minute under the standard assay condition.

### **Effect of other parameter on Lipase activity**

Effect of temperature on the catalytic activity of lipases was determined by measuring the enzyme activity at temperature ranging between 40°C - 65°C under the standard assay

conditions. Effect of pH on enzyme activity was determined by measuring the enzyme activity at different pH values ranging from 3 to 9 by using suitable buffers like 50 mM sodium acetate (pH 5 and 6), 50 mM sodium phosphate (pH 7 and 8), 50 mM glycine-NaOH buffer (pH 9 and 10), Sodium bicarbonate - NaOH buffer (pH 11) respectively. Optimum NaCl concentration was determined by using different concentration of NaCl ranging from 0.5 to 4.5% in the production medium. The enzyme activity was also examined at different incubation period (24 to 120hours) (Bisht and Panda, 2011).

## RESULTS AND DISCUSSION

Thermophilic bacteria have peculiar molecular level modifications at cellular and subcellular level to survive at high temperatures and dry climate as of Rajasthan, India. Thermophiles and hyperthermophiles secrete such enzymes which are thermostable and display irreversible protein denaturation at high temperatures. Total 16 bacterial strains were isolated from the soil of arid and semi-arid region of Rajasthan.

### Identification of the lipolytic enzyme producing thermophilic bacteria:

Out of 16 bacterial isolates only two strains (J1 and J3) were screened as a potent degrader of lipid by producing lipolytic enzyme and showed clear lipolytic zone on tributyrin agar plate at 50°C (figure 1).



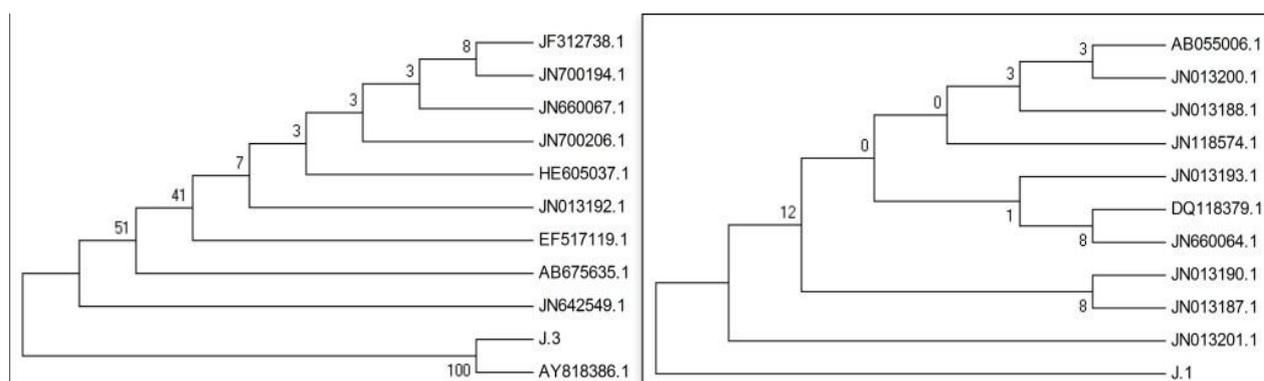
**Figure 1** Screening of lipolytic enzyme producing thermophilic bacteria at 50°C, clear zone indicates the hydrolysis of Tributyrin as a result of lipase production

These two bacterial isolates were characterized on the basis of cultural characteristics, microscopic appearance, biochemical tests (Table 1) and molecular analysis.

**Table 1** Characterization of J1 and J3 bacterial strains

<b>Mode of characterization</b>	<b>Characteristics of bacterial isolates</b>	<b>J1 bacterial strain (<i>B. licheniformis</i>)</b>	<b>J3 bacterial strain (<i>B. subtilis</i>)</b>
Cultural characteristics	Colony morphology on nutrient agar	Irregular, mucoid, creamy yellow and fast growing colonies	Small, round, regular, mucoid, creamy yellow and fast growing colonies
Microscopic characteristics	Spore staining, Gram staining and Motility	Spore forming and Gram positive	Spore forming, Gram positive and motile
Biochemical characteristics	Indole test	Negative	Negative
	Methyl Red test	Negative	Negative
	Voges Proskauer test	Positive	Positive
	Citrate utilization test	Positive	Positive
	Catalase test	Positive	Positive
	Oxidase test	Positive	Positive
	Urease test	Negative	Negative
	Nitrate reduction test	Positive	Positive
	Gelatin liquefaction test	Positive	Positive
	Starch hydrolysis test	Positive	Positive
	Hydrogen sulphide test	Negative	Negative
	Hydrogen peroxidase test	Positive	Positive
	Casein hydrolysis test	Positive	Positive
	Glucose fermentation	Negative	Negative

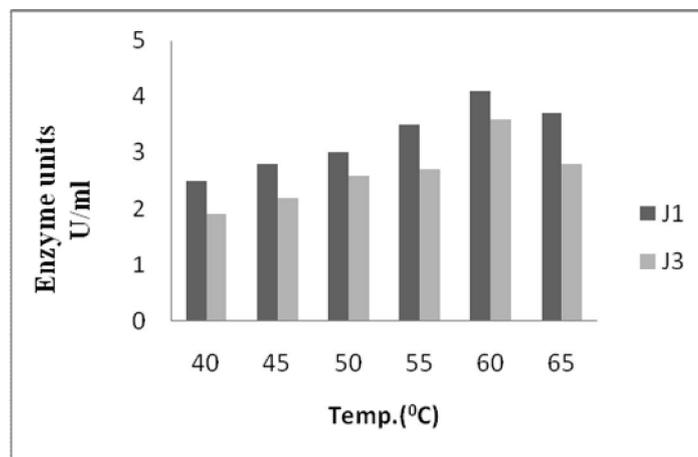
Molecular characterization of these strains was done by DNA isolation (CTAB method) and 16S rDNA analysis. These amplified 16S rDNA sequences of the bacterial strains were aligned using online tool (MEGA 4). The taxonomical identification done by the phylogenetic tree construction as followed by the comparison of these bacterial sequences with other homologous bacterial sequences. After morphological, physiological, biochemical and taxonomical identification, these two bacterial isolates were identified as *Bacillus licheniformis* (J1) and *Bacillus subtilis* (J3) (figure 2).



**Figure 2** Phylogenetic tree of *Bacillus* sp. J1 and J3

### Effect of Temperature on the lipolytic activity

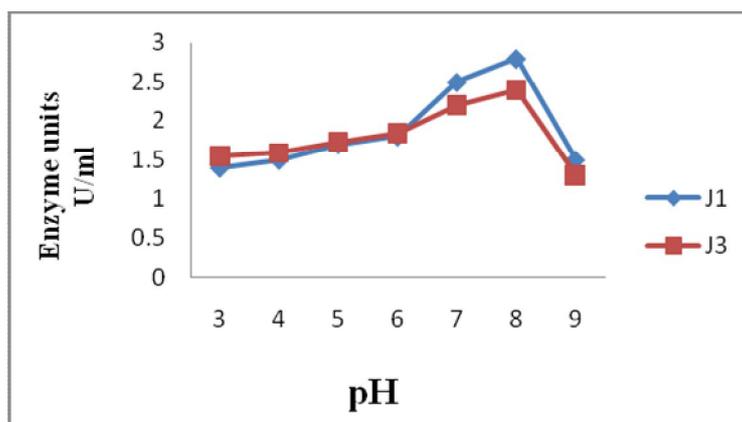
The effect of temperature on the activity of crude enzyme was determined at various temperature ranging from 40°C to 65°C at pH 7. J1 and J3 bacterial strains showed good enzyme activity between 60°C to 65°C. The optimum enzyme production was at 60°C and reduction was observed in enzyme activity above or below 60°C (figure 3). At 60° J1 and J3 showed maximum 4.1 and 3.5 enzyme unit (U/ml) respectively.



**Figure 3** Activity of Lipase at different temperature

### Effect of pH on the lipolytic activity

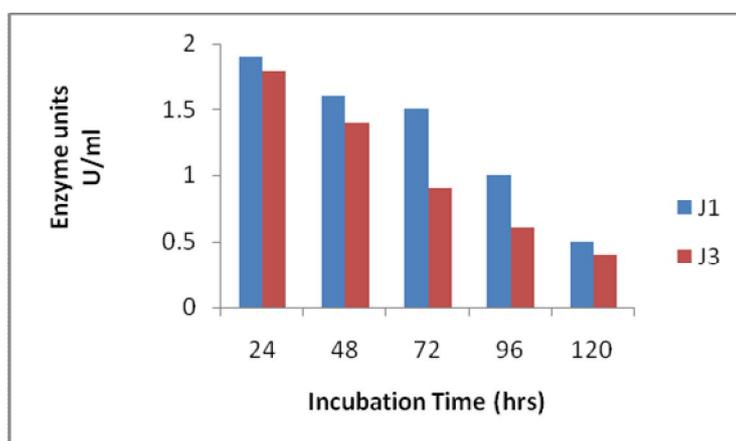
The effect of the pH on the crude lipolytic enzyme activity of *Bacillus* sp. was examined at various pH ranging from 3 to 9 (figure 4). Broad range of enzyme activity was observed (pH 3-9). The optimum pH 8 was recorded, which was close to the optimum pH value of most *Bacillus* lipases (Haki and Rakshit, 2003). At pH 8 J1 and J3 showed maximum enzyme unit 2.9 and 2.4 U/ml respectively.



**Figure 4** Activity of Lipase at different pH

### Effect of time duration on production of the lipolytic activity

Maximum production of lipolytic enzyme by *Bacillus licheniformis* (J1) and *Bacillus subtilis* (J3) was recorded at 24 hours at 60°C, further there was gradual decrease in growth of the organism and enzyme production. The growth rate on 120 hours (5<sup>th</sup> day) was very low. These organisms did not show enzyme production after 120 hrs. 1.9 U/ml and 1.8 U/ml maximum enzymatic activity was observed by J1 and J3 bacterial strains respectively at 24 hrs incubation period (figure 5).

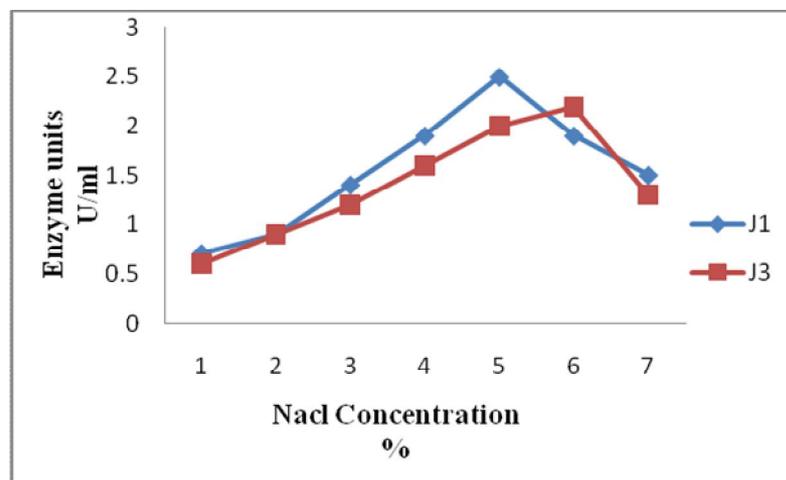


**Figure 5** Activity of Lipase at different Incubation period

### Effect of NaCl concentration on lipolytic activity:

At 5% NaCl concentration the enzyme activity was found optimum. As the salt concentration was increased or decreased, a gradual decrement was recorded in enzyme

production, enzyme activity and growth of organisms. For J1 bacterial strain maximum enzymatic activity was 2.5 U/ml and for J3 strain maximum activity was 2.0 U/ml at 6 % salt concentration (figure 6).



**Figure 6** Activity of lypolytic enzyme at different NaCl concentration.

**Bisht and Panda, 2011** isolated and characterized thermophilic lipase producing bacteria from an Indian hot spring, named as AK-P1, AK-P2 and AK-P3 respectively. The optimum temperature and pH for this crude enzyme activity was 60°C and 10 pH. They found the strain AK-P1 had highest homology (100%) with *Acinetobacter* sp. 01B0, AK-P2 had highest homology (99.9%) with *Brevibacillus borstelensis* and AK-P3 have shown 99.2 % similarities with *Porphyrobacter cryptus*. **Cho et al., 2000** studied thermostable lypolytic enzyme of *Bacillus thermoleovorans* ID-1. The optimum temperature of the lipase was 75°C, which is higher than other known *Bacillus* lipases. For expression in *Escherichia coli*, the lipase gene was subcloned in pET-22b (+) vector with a strong T7 promoter. **Cho et al., 2000** recorded approximately 1.4-fold greater lipase activity than under the native promoter. **Rahman et al., 2007** performed research on a thermophilic lipolytic bacterium, *Geobacillus zalihae* sp. nov. This species was isolated from palm oil mill effluent in Malaysia. From their study they concluded that Strain T1<sup>T</sup> was able to secrete extracellular thermostable lipase into culture medium. This T1<sup>T</sup> strain was identified as *Geobacillus zalihae*. This strain was different from the other related strains *Geobacillus kaustophilus* (DSM 7263T) and *Geobacillus thermoleovorans* (DSM 5366T) because of few differences in physiological character, cellular fatty acids composition, RiboPrint analysis, length of lipase gene and protein profile. In present investigation, we isolated *Bacillus* sp from soil which show

lypolytic enzyme activity at high temperature. To increase the enzyme activity, we optimized physical and cultural conditions (Temperature, pH NaCl and incubation time). After optimization of cultural conditions, these bacterial strains showed significant increased activity of desired enzyme.

## CONCLUSION

Lypolytic enzyme are most useful enzymes known and having great significance. These enzymes have potential applications in food, pharmaceutical and fine chemical industries. Microbial production of Lipase from *B. licheniformis* and *B. subtilis* is very significant, because it is cost efficient, less time consuming and convenient to manage. This study reports biological production of thermostable lypolytic enzymes by soil bacteria *B. licheniformis* and *B. subtilis* isolated from arid and semi-arid region of Rajasthan. In present study optimum enzyme activity of lipolytic enzymes were recorded at 60°C temperature, 5 % and 6% NaCl concentrations, and pH 8.0 by J1 (*B. licheniformis*) and J3 (*B. subtilis*) sp. respectively. At the optimum conditions *B. licheniformis* and *B. subtilis* will also produce higher amount of lipolytic enzymes per liter of culture broth, this amount of enzyme higher than before optimization. After comparison with other studies and research we are able to conclude that thermostable lypolytic enzymes producing bacterial strains which degrade lipid substrate and shows catalytic activity at high temperature.

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